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# The mutagenic mechanism of oxygenated alkylhydrazones occurs through alkyl radicals and alkyldiazonium ions†

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Acetone alkylhydrazones have been reported to be mutagenic in *Salmonella typhimurium* TA1535 after exposure to oxygen, and the corresponding 2-alkylazo-2-propyl hydroperoxides are formed by autoxidation as a result. The aims of this study were to investigate the mutagenic mechanisms of a methyl analogue, 2-methylazo-2-propyl hydroperoxide (MAPH), by comparing the mutagenic potency of specific *Salmonella* strains, detecting the DNA adducts that cause mutagenicity, and observing the hydroxyl radical and methyl radical with the electron spin resonance (ESR) spin-trapping method. MAPH showed stronger mutagenicity in both *Salmonella typhimurium* YG3001, a strain sensitive to hydroxyl radicals, and *Salmonella typhimurium* YG7108, a strain sensitive to alkylating agents, than the original *Salmonella typhimurium* TA1535 strain. Moreover, MAPH resulted in the formation of 8-hydroxy-2'-deoxyguanosine and O<sup>6</sup>-methyl-2'-deoxyguanosine in a reaction with DNA. These results showed that the mutagenicity of hydrazones was ascribed to the generation of reactive species by autoxidation, namely that of the alkyldiazonium ion and also the hydroxyl radical.

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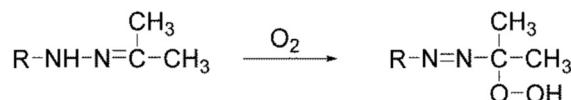
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## Introduction

Hydrazones and their derivatives are known to exhibit a wide range of biological activities, including antioxidant, anti-inflammatory, anticonvulsant, analgesic, antimicrobial, anticancer, antiprotazoal, antioxidant, antiparasitic, antiplatelet, cardioprotective, anthelmintic, antidiabetic, antitubercular, trypanocidal, and anti-HIV activities.<sup>1–4</sup> In addition, hydrazones and their derivatives have been used as synthetic intermediates for drugs but are known to be mutagenic and carcinogenic.<sup>5–7</sup> In particular, hydrazones have received increasing attention because they are present as impurities in active pharmacological ingredients.<sup>8</sup> Although the toxic mechanism of hydrazines has been well studied, the mutagenic mechanism of hydrazones has not been studied. Therefore, it is important to elucidate the mutagenic mechanism of hydrazones.

We previously reported that acetone alkylhydrazones were mutagenic in *Salmonella typhimurium* TA1535 and *Escherichia*



R = methyl, ethyl, propyl, butyl

**Fig. 1** The formation of 2-alkylazo-2-propyl hydroperoxides from acetone alkylhydrazones.

*coli* WP2<sup>hcr</sup> after a dichloromethane solution of acetone alkylhydrazones was bubbled with oxygen gas and the products were characterized as 2-alkylazo-2-propyl hydroperoxides by nuclear magnetic resonance (NMR) analysis (Fig. 1).<sup>9</sup> In the present study, the mutagenic mechanisms of 2-methylazo-2-propyl hydroperoxide (MAPH) were elucidated by comparing the *Salmonella typhimurium* TA1535, YG3001 and YG7108 strains and detecting the DNA adducts and radical species derived from MAPH in aqueous solution (pH 7.4).

## Materials and methods

### Chemicals

All chemicals were of the highest quality available. Methylhydrazine sulfate, acetone, nuclease P1, and 8-hydroxy-2'-deoxyguanosine (8-OHdG) were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). 5,5-Dimethylpyrrolidine

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*N*-oxide (DMPO) was obtained from Dojindo (Kumamoto, Japan). Bacterial alkaline phosphatase was purchased from Toyobo (Osaka, Japan). Calf thymus DNA and *O*<sup>6</sup>-methyl-2'-deoxyguanosine (*O*<sup>6</sup>-MedG) were obtained from Sigma-Aldrich (St Louis, MO, USA). The calf thymus DNA was purified by phenol-chloroform and ethanol precipitation.

### Preparation of MAPH

Methylhydrazine was stirred at 60 °C for 2 h in acetone under an N<sub>2</sub> atmosphere. The mixture was neutralized with 30% NaOH, and the mixture was extracted with dichloromethane (30 mL × 3). The dichloromethane layer was dried over anhydrous sodium sulfate and filtered, and then the dichloromethane was evaporated under reduced pressure. The residue was distilled under reduced pressure (bp.<sub>33</sub> 42 °C) to purify acetone methylhydrazone.<sup>9</sup>

A solution of acetone methylhydrazone in dichloromethane was bubbled with oxygen gas at approximately 25 mL min<sup>-1</sup> at room temperature. After 2 h, the reaction mixture was added to acetonitrile and was evaporated under reduced pressure to remove the dichloromethane. This afforded a tan-yellow acetonitrile solution. MAPH appeared to be explosive and was treated with maximum precaution. Condensation and/or extensive purification by distillation was omitted. <sup>1</sup>H-NMR (CDCl<sub>3</sub>) δ ppm: 1.46 (s, 6H), 3.82 (s, 3H), and 9.19 (s, 1H).

### Mutagenicity assay

The mutagenicity assay was based on the method described by Ames.<sup>10</sup> Dr T. Nohmi (National Institute of Health Sciences, Tokyo, Japan) kindly provided the *Salmonella typhimurium* TA1535, *Salmonella typhimurium* YG3001 and *Salmonella typhimurium* YG7108 strains.

### Treatment of DNA with MAPH

Calf thymus DNA (0.5 mg/0.5 mL) was incubated with MAPH at 37 °C for 2 h. After the addition of 5 M NaCl, the DNA was precipitated with ethanol. Then, the DNA was dissolved in 1 mM EDTA at 95 °C for 5 min to denature the double-stranded DNA into single-stranded DNA. Then, the solution was cooled immediately in an ice-water bath. The DNA was digested to deoxynucleotides by incubation first with nuclease P1 at 37 °C for 1 h and then with bacterial alkaline phosphatase at 37 °C for 1 h in 0.1 M Tris-HCl buffer (pH 7.5). The mixture was centrifuged (15 000 rpm, 3 min, and 4 °C), and the upper layer was separated and analyzed for the 8-OHdG and *O*<sup>6</sup>-MedG contents by HPLC.<sup>11</sup>

### Analysis of 8-OHdG

The amounts of 8-OHdG and dG in the digested DNA were analyzed using HPLC (Shiseido NANOSPACE SI-1 2001) with a UV detector (Shiseido NANOSPACE SI-1 2002 at 254 nm) and an electrochemical detector (Shiseido NANOSPACE SI-1 2005). The separation conditions were as follows: column, Shiseido Capcell Pak C<sub>18</sub> (1.5 × 250 mm); column temperature, 40 °C; mobile phase, 50 mM potassium phosphate (pH 5.5) containing 10% methanol; and flow rate, 0.6 mL min<sup>-1</sup>. The level of

8-OHdG in the DNA was expressed as the number of 8-OHdG molecules per 10<sup>2</sup> dG.<sup>12</sup>

### Analysis of *O*<sup>6</sup>-MedG

The amount of *O*<sup>6</sup>-MedG in the digested DNA was analyzed by HPLC with a UV detector. The separation conditions were as follows: column, Shiseido Capcell Pak C<sub>18</sub> (1.5 × 250 mm); column temperature, 40 °C; mobile phase, 60 min gradient of 5–50% methanol in 0.1 M sodium phosphate buffer (pH 7.4); and flow rate, 0.5 mL min<sup>-1</sup>. The level of *O*<sup>6</sup>-MedG in the DNA was expressed as the number of *O*<sup>6</sup>-MedG molecules per 10<sup>2</sup> dG.

### ESR measurement by spin trapping

The ESR spectra were recorded in a flat cell using a JEOL JES-RE3X spectrometer. The reactions were initiated by mixing 120 μL of 0.1 M sodium phosphate buffer (pH 7.4), 10 μL of 5.7 M MAPH and 20 μL of a spin-trapping agent at room temperature, and the spectra were obtained after mixing for 1 min. The typical instrumental parameters were as follows: power, 10 mW; field, 336.0 ± 7.5 mT; modulation, 0.063 mT; time constant, 0.3 s; sweep time, 2 min; and room temperature.

## Results and discussion

### Mutagenicity of MAPH in *Salmonella typhimurium* strains

In our previous study, 2-alkylazo-2-propyl hydroperoxides were shown to be mutagenic in *Salmonella typhimurium* TA1535, TA100, and TA102 and *Escherichia coli* WP2hcr<sup>-</sup>.<sup>9</sup> The Ames mutagenicity assay is used widely to assess the mutagenic potential of chemical compounds, and it is also a useful tool for the investigation of mutagenic mechanisms by comparing the activity of strains with various deficiencies in repair systems.<sup>10,13</sup> *Salmonella typhimurium* YG3001 and YG7108, which were derived from *Salmonella typhimurium* TA1535, were used in this study. *Salmonella typhimurium* YG3001 is highly sensitive to reactive oxygen species because of its deficiency in 8-hydroxyguanine DNA glycosylase in the *Salmonella typhimurium* TA1535 parent strain.<sup>14</sup> *Salmonella typhimurium* YG7108 is highly sensitive to alkylating agents because of its deficiency in *O*<sup>6</sup>-methylguanine DNA methyltransferase in the *Salmonella typhimurium* TA1535 parent strain.<sup>15</sup> The mutagenicity of MAPH was detected in three strains, and the activity increased in a dose-dependent manner with the concentration of MAPH (Fig. 2). The mutagenic potency was stronger in both *Salmonella typhimurium* YG3001 and *Salmonella typhimurium* YG7108 than in *Salmonella typhimurium* TA1535. The data indicated that 8-OHdG and *O*<sup>6</sup>-MedG caused mutagenic effects through base substitution mutations in *Salmonella typhimurium* YG3001 and YG7108, respectively.<sup>16–18</sup>

### Formation of 8-OHdG and *O*<sup>6</sup>-MedG in DNA treated with MAPH

To confirm the mutagenic mechanism, 8-OHdG and *O*<sup>6</sup>-MedG were quantified by high-performance liquid chromatography (HPLC) after treating MAPH with DNA (Fig. 3). The levels of

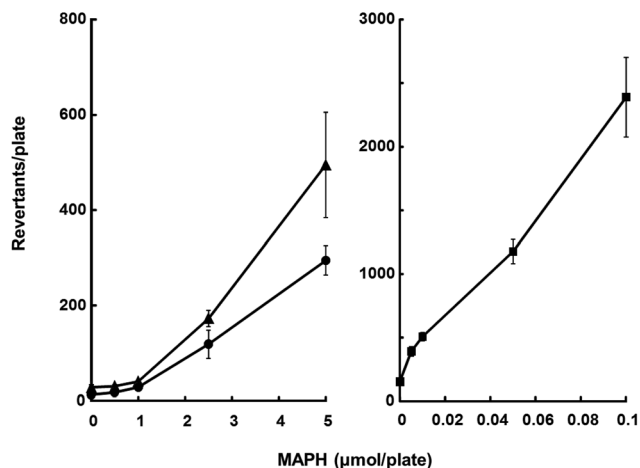


Fig. 2 The mutagenicity of MAPH in *Salmonella typhimurium* TA1535 (●), YG3001 (▲) and YG7108 (■).

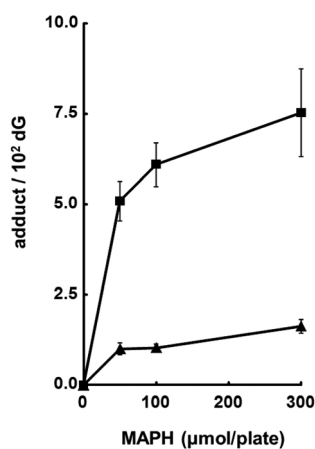


Fig. 3 The formation of 8-OHdG (▲) and O<sup>6</sup>-MedG (■) from the reaction of MAPH and DNA.

both of the DNA adducts increased in a dose-dependent manner with the concentration of MAPH. The amount of O<sup>6</sup>-MedG formed was higher than 8-OHdG. The data agreed well with the mutagenic potency, in which the mutagenicity of MAPH in *Salmonella typhimurium* YG7108 was higher than that in *Salmonella typhimurium* YG3001.

#### Detection of radical species by ESR spin trapping measurements

Electron spin resonance (ESR) is a conventional technique capable of directly detecting radical species. The high reactivity and short lifetimes of free radicals usually make them very unstable, particularly in biological systems, thus rendering them undetectable by ESR. In the ESR spin-trapping technique, free radicals with a short lifetime are allowed to react with spin-trapping agents to form a more stable radical spin-trapping adduct with a longer lifetime.<sup>19</sup> The ESR spectrum of

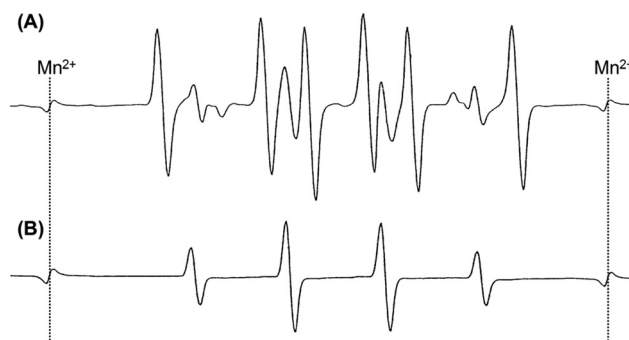


Fig. 4 (A) The ESR spectrum of MAPH in phosphate buffer (pH 7.4) in the presence of DMPO. (B) The ESR spectrum of the Fenton reagent in phosphate buffer (pH 7.4) in the presence of DMPO.

the spin adduct yields specific information to identify the original free radical.<sup>20</sup>

The production of free radicals from MAPH in aqueous solution was demonstrated using the ESR technique of spin trapping with 5,5-dimethylpyrroline *N*-oxide (DMPO). The typical 1:2:2:1 ESR signal of the DMPO-OH adduct was observed, as shown in Fig. 4. The spectrum was identical to the spectrum of the Fenton reagent (Fe<sup>2+</sup> and H<sub>2</sub>O<sub>2</sub>), which is often used as a hydroxyl radical generation system.<sup>21</sup> In addition, the formation of the DMPO-OH adduct was diminished by the addition of ethanol or sodium ascorbate, which are known hydroxyl radical scavengers (data not shown). The data indicated that a hydroxyl radical was formed from MAPH and then reacted with dG to form 8-OHdG.

As shown in Fig. 4, an unknown species with 6 signals derived from the reaction with DMPO and a carbon-centered radical was present together with the DMPO-OH signal. To identify the unknown radical species, 3,5-dibromo-4-nitrosobenzenesulfonate (DBNBS) was used as a spin-trapping agent instead of DMPO (Fig. 5A). The spectrum was identical to the spectrum of DMSO with the Fenton reagent,<sup>22</sup> indicating that the DBNBS-CH<sub>3</sub> adduct was formed from MAPH (Fig. 5B).

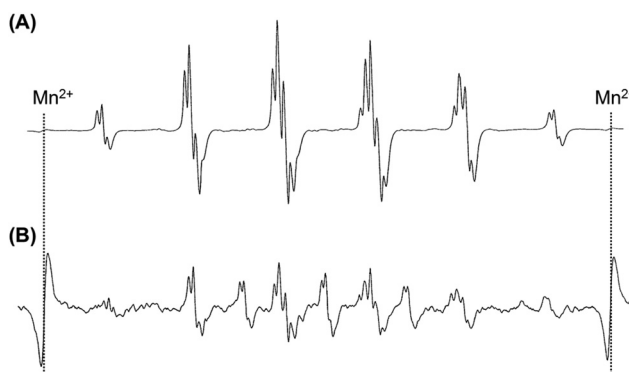


Fig. 5 (A) The ESR spectrum of MAPH in phosphate buffer (pH 7.4) in the presence of DBNBS. (B) The ESR spectrum of DMSO with the Fenton reagent in phosphate buffer (pH 7.4) in the presence of DBNBS.

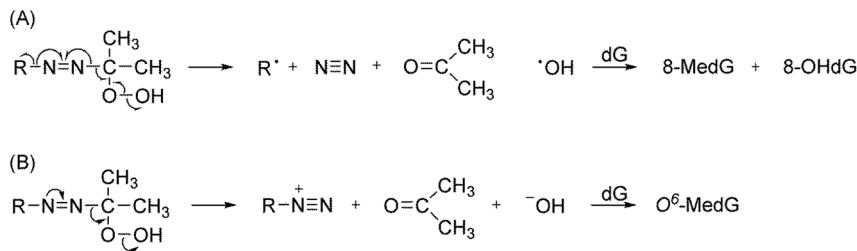


Fig. 6 The decomposition mechanisms of MAPH.

Thus, MAPH decomposed in aqueous solution (pH 7.4) to generate hydroxyl radicals and methyl radicals simultaneously.

Methyl radicals have been reported to be produced from *t*-butyl hydroperoxide/ $\text{Fe}^{2+}$ ,<sup>23,24</sup> cumene hydroperoxide/ $\text{Fe}^{2+}$ ,<sup>25</sup> and DMSO or methionine sulfoxide/Fenton reagents,<sup>26,27</sup> as well as *via* the metabolism of 1,2-dimethylhydrazine and procarbazine.<sup>28</sup> Although methyl radicals are less reactive than hydroxyl radicals, they can abstract hydrogen atoms and cause DNA strand cleavage.<sup>29,30</sup> Furthermore, methyl radicals alkylate DNA to form 5-methyl-2'-deoxycytidine (5-MedC), 8-methyl-2'-deoxyguanosine (8-MedG) and 8-methyl-2'-deoxyadenosine (8-MedA).<sup>31</sup> 5-MedC is known to be involved in the initial step of epigenetic changes,<sup>27</sup> while 8-MedG has been reported to stabilize the *Z* conformation of short oligonucleotides.<sup>32</sup> Although the mutagenic properties of 8-MedG and 8-MedA are unclear, their DNA adducts can lead to DNA depurination which is known to be mutagenic.<sup>30</sup> In the Ames *Salmonella* mutagenicity assay, it was proposed that 8-MedG and 8-MedA did not cause mutagenicity in *Salmonella typhimurium* TA1535, YG3001 and YG7108. The roles of DNA methylation by methyl radicals in carcinogenesis and mutagenesis must therefore be explored further.

A decomposition mechanism for MAPH through homolytic and heterolytic pathways has been proposed, as shown in Fig. 6. During homolytic cleavage, methyl radicals and hydroxyl radicals were formed, along with acetone and nitrogen gas, and the methyl radical and hydroxyl radical reacted with dG to form 8-MedG and 8-OHdG, respectively. During heterolysis, the alkyldiazonium ion was formed, along with acetone and a hydroxide ion. The alkyldiazonium ion reacts with dG to form  $\text{O}^6$ -alkyldG.

MAPH decomposed in aqueous solution through two pathways, and the mutagenicity of MAPH was derived from the alkylating species and also the reactive oxygen species. The formation of hydrazone hydroperoxides by autoxidation could be involved in the mutagenic mechanism of the hydrazones.

It has been reported that hydrazine reacts with endogenous formaldehyde to form formaldehyde hydrazone, and this intermediate can be metabolized to the potent methylating agent, the methyl-diazonium ion.<sup>33,34</sup> In experiments using the post-mitochondrial (S9), microsomal, cytosolic or mitochondrial cell fractions from rat liver *in vitro*, the methylation of DNA guanine occurred, and S9 was the most active fraction. Bovine liver catalase and heme-containing cytochrome readily trans-

formed hydrazine/formaldehyde into a methylating agent. This pathway suggests that formaldehyde hydrazone was oxidized by catalase or a similar enzyme to form an *N*-hydroxy formaldehyde hydrazone, which can rearrange to the unstable methyl-diazonium ion through the loss of water and rapidly react with cellular nucleophiles, such as DNA.<sup>34</sup>

Gericke *et al.* reported that the mutagenicity of methyl-hydrazones was not correlated to their alkylating behavior for 4-(4-nitrobenzyl)pyridine.<sup>6</sup> Thus, their mutagenic activity might involve radical species, especially reactive oxygen species.

Acetone enters air, water, and soil as a result of natural processes and human activities. Acetone occurs naturally in plants, trees, volcanic gases, and forest fires. Humans breathe out acetone produced from the natural breakdown of body fat. Acetone is also released during its manufacture and use, in exhaust from automobiles, and from tobacco smoke, landfills, and certain types of burning waste.<sup>35</sup>

Hydrazines constitute an important class of compounds that humans are often exposed to because they are natural components of mushrooms and tobacco, and they are also constituents of herbicides, rocket fuel, and drugs.<sup>8</sup> Thus, there is a possibility that hydrazines may react with endogenous acetone to form a condensation product that could cause the formation of methylating agents and hydroxyl radicals.

## Conclusion

This is the first study to show that hydrazone hydroperoxides were generated by autoxidation and their mutagenicity was derived from the alkyldiazonium ion and the radical species. Overall, the radical species were responsible, in part, for the mutagenicity of the hydrazones. Moreover, by altering the reaction conditions, it may be possible to control the MAPH degradation pathways, the alkyldiazonium ion pathway and the radical pathway.

## Abbreviations

DBNBS	3,5-Dibromo-4-nitrosobenzenesulfonate
DMPO	5,5-Dimethylpyrrolidine <i>N</i> -oxide
ESR	Electron spin resonance

8-OHdG	8-Hydroxy-2'-deoxyguanosine
MAPH	2-Methylazo-2-propyl hydroperoxide
8-MedA	8-Methyl-2'-deoxyadenosine
5-MedC	5-Methyl-2'-deoxycytidine
O <sup>6</sup> -MedG	O <sup>6</sup> -Methyl-2'-deoxyguanosine
8-MedG	8-Methyl-2'-deoxyguanosine.

## Conflict of interest

There are no conflicts of interest to declare.

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